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TITLE: Enhancing the Immunogenicity of a Dengue-2 DNA Vaccine  
with Adjuvants and Anti-FCγRI Antibodies

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## INTRODUCTION

Department of Defense (DoD) researchers have used the DNA vaccine approach to develop experimental vaccines against DEN-1 and DEN-2. These vaccines contain the envelope (E) and premembrane (prM) genes of the viruses. The E gene was chosen for inclusion in the DNA vaccines because monoclonal antibody mapping and recombinant protein immunization studies with flaviviruses have shown that the E gene contains the major epitopes responsible for eliciting neutralizing antibodies. The prM gene was also included in the vaccine because the expressed protein is thought to prevent low pH-induced irreversible conformational changes in the E protein as it is processed through the acidic compartments of the Golgi complex prior to secretion. The DEN-2 and DEN-1 DNA vaccines have been tested in mice and shown to elicit neutralizing antibody responses.

Two primate DNA immunization/challenge studies were completed. One utilized Rhesus monkeys, the other *Aotus* monkeys. Both studies were very similar in design and outcome. One-third of the animals were injected with control DNA (plasmid that does not express prM/E) and two-thirds of the animals were immunized with the DNA vaccine that expresses the prM/E genes of DEN-1 virus. All of the animals that received the DEN-1 DNA vaccine responded by producing neutralizing antibodies. The antibody levels peaked one month after the last DNA boost and then steadily declined during the following five months at which time the animals were challenged with DEN-1 virus. Upon challenge with live dengue virus, the DEN-1 DNA vaccine-immunized monkeys showed a significant reduction in the mean days of viremia compared to naïve controls, but the protection was not complete. Although successful, these studies do indicate the need to improve the efficacy of DEN DNA vaccines.

Immune responses to DNA vaccines have been enhanced by co-administering them with immune-modulating compounds including cytokines, chemokines, co-stimulatory molecules and cationic lipids. Although these approaches have resulted in improved potency, none of these are approved for use in humans with prophylactic vaccines. Aluminum-based adjuvants have been used successfully in humans to enhance the immune response to protein vaccines and are approved for use in humans. Studies have demonstrated that intramuscular co-administration of DNA vaccines with aluminum phosphate enhanced the antibody response 10- to 100-fold. This response occurred with DNA vaccines for hepatitis B and influenza A. The use of calcium phosphate, aluminum hydroxide and aluminum hydrophosphate as adjuvants had an inhibitory effect on the antibody response to the DNA vaccines. This inhibitory effect was the result of the DNA binding to these adjuvants, making less available for uptake by antigen presenting cells (APCs). The enhancement of vaccine potency was the result of increased recruitment of APCs.

Highly immunogenic carriers such as tetanus toxoid, diphtheria toxoid and cholera toxin have been used successfully to enhance the immunogenicity of polysaccharide vaccines. These proteins work by involving T-cells in the processing of the carrier protein (T-cell help) and by generating both T- and B-cell memory. Little is known about the effect of these carrier proteins on the immune response to DNA vaccines.

Another possible way to enhance the uptake, expression and processing of vaccines is to link them with antibodies that more efficiently direct them to APCs. Gosselin et al. cross-linked anti-

FcγRI monoclonal antibodies with tetanus toxoid and compared the immunogenicity of this compound with that of tetanus toxoid alone. The results showed that by linking the immunogen with the monoclonal antibody, antigen presentation was increased 100-fold. The mechanism of the increased antibody response was thought to involve the more efficient uptake and processing of the tetanus toxoid by APCs. This approach has yet to be attempted using DNA vaccines as the immunogen. Because only a small portion of DNA vaccines is actually taken up and expressed by APCs, it stands to reason that increasing the efficiency of uptake would result in enhanced cellular and humoral immune responses to nucleic acid vaccines.

This proposal seeks to enhance the immune response to a dengue (DEN) prM/E DNA vaccine by utilizing aluminum phosphate, tetanus toxoid and anti-FcγR monoclonal antibodies as adjuvants in various formulations. Phase I of the project will be conducted in mice to demonstrate the feasibility of enhancing anti-DEN immune responses with these novel approaches. Phase II will be conducted using non-human primates to evaluate the protective efficacy of DNA vaccine formulations that are shown to be immunogenic in mice.

## BODY

### Year 1

#### Summary Statement of Work:

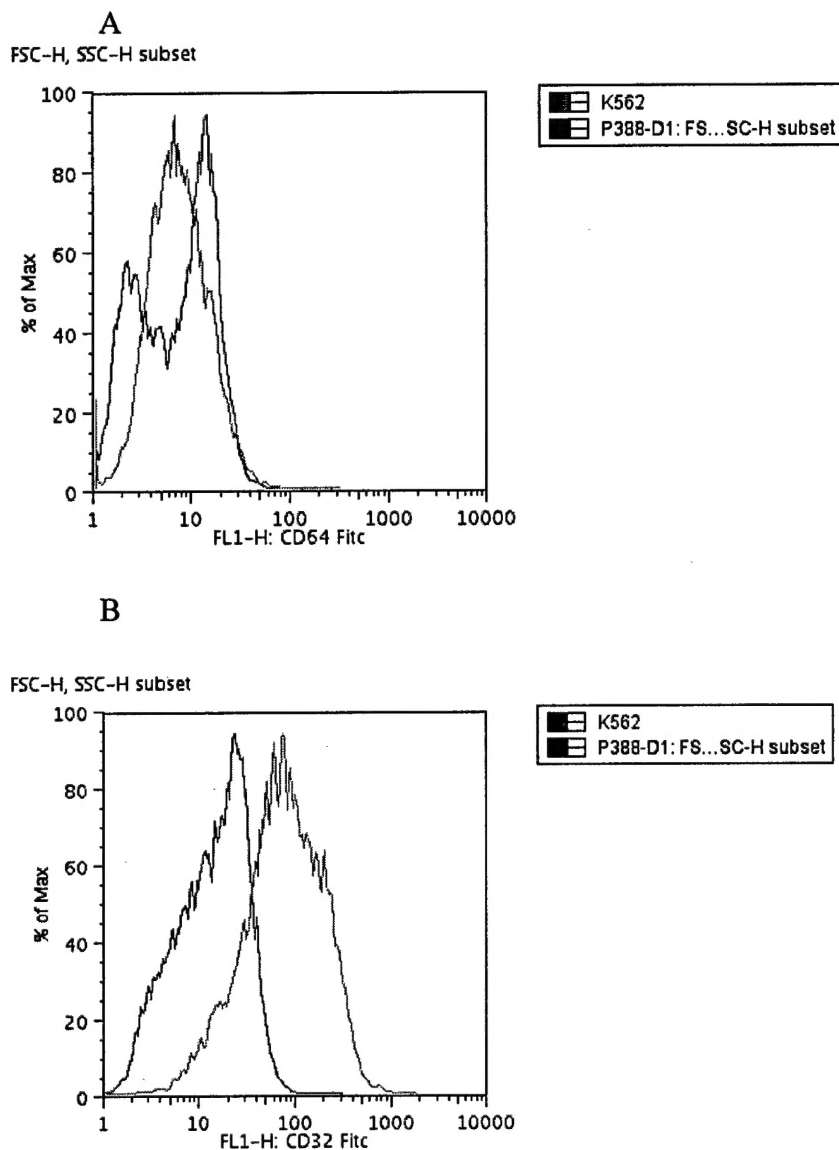
1. DNA vaccine is chemically linked with FcγRI antibody and evaluated for expression *in vitro*.
2. Co-immunize mice with DNA vaccine and aluminum phosphate and tetanus toxoid. Immunize mice with DNA vaccine linked with anti-FcγRI antibody.
3. Analyze antibody and cellular immune responses to the adjuvanted DNA vaccines.

#### Progress:

1. DNA vaccine linkage to FcγRI antibody

The first task was to link the dengue DNA vaccine to anti-FcγR monoclonal antibodies. Prior to performing the chemical linking procedure, we first sought to confirm that the monoclonal antibodies selected for linkage to the DNA vaccine recognize the FcγR of mammalian immune cells. The first set of anti-FcγR antibodies, consisted of anti-FcγRII (CD32) and anti-FcγRIII (CD16) (Accurate Chemical, Westbury, NY). Indirect immunofluorescence assays (IFA) were performed to evaluate the recognition of Fcγ receptors on K562 cells (human monocyte cell line) and P388D1 cells (mouse monocyte cell line). Experimental results indicated poor recognition of mouse and human Fcγ receptors by these monoclonal antibodies, as indicated by low level immunofluorescence. A second set of monoclonal antibodies was obtained (Pharmigen, Inc. San Diego CA) and consisted of anti-FcγRI and anti-FcγRII. IFA demonstrated recognition of anti-FcγRII receptors but not FcγRI (CD64) on K562 cells. The P388D1 mouse cells showed no reactivity with either antibody. This pattern of immunoreactivity was confirmed by flow cytometry analysis (Figure 1A&B).

Figure 1



Further studies to find P388D1 immunoreactive anti FcγR monoclonal antibodies were conducted. Use of another commercial product, with combined anti- FcγRII and anti- FcγRIII immunoreactivity, revealed positive results against P388D1 cells by IFA.

Experiments to link the FcγRII/III monoclonal antibody to DEN-1 DNA vaccine were then conducted. The DEN-1 DNA vaccine was used instead of the DEN-2 vaccine as originally planned because more extensive non-human primate efficacy data were available for the DEN-1 vaccine. Much of these DEN-1 DNA vaccine data became available after the original proposal

was submitted. Historical comparisons with the dengue 1 DNA vaccine data would allow for a more rigorous determination of whether the modifications improve the immunogenicity and protective efficacy of the DNA vaccine.

To conjugate the anti-FcγRII/RIII monoclonal antibody to the DNA vaccine, the DNA was linearized by Xho I restriction enzyme digestion to produce "sticky" 5' and 3' ends. To first confirm that the linearized DNA is capable of expressing the DEN-1 prM and E genes, 293 cells were transfected with the linearized DNA and analyzed by IFA using the anti-DEN monoclonal antibody 4G2. 293 cells are routinely used to evaluate expression of our experimental DNA vaccine constructs. These cells were used initially so that a head-to-head comparison can be made between the levels of expression obtained with the circular versus the linearized DNA vaccine plasmid construct. The results showed that the linearized DNA efficiently expressed the DEN-1 genes and that the level of expression was equivalent to that seen with the closed circular plasmid construct. Since our plan was to use the P388D1 cells to evaluate in vitro uptake and expression of the DNA vaccine/anti-FcγR antibody fusion product, we next wanted to confirm that the P388D1 cells were first capable of efficiently expressing the prM and E genes of the linearized DEN 1 plasmid before proceeding with the conjugation. Despite repeated attempts, we were unable to efficiently transfect the P388D1 cells and thus could not evaluate the expression of the linear DNA in this cell line. Another mouse macrophage/monocyte cell line (Raw 264.7 cells) was then evaluated. Raw 264.7 cells are transformed by Abelson murine leukemia virus. Following transfection with the DEN-1 vaccine plasmid, the Raw 264.7 cells expressed the prM and E proteins at a level similar to that of the 293 cells. Repeat binding studies with the anti-FcγRII/RIII monoclonal antibody showed efficient binding to the Raw 264.7 cells by IFA.

The most efficient way to combine the DNA to the antibody is via thiol linkage to the amine groups on the antibody. The first step was to add a thiol group to the linearized DNA vaccine. To accomplish this, T4 DNA polymerase was used to incorporate dASTP (2'-deoxyadenosine 5'-O-(1-thiotriphosphate) into the ends of the linearized DNA. The thiol-DNA was then incubated with the heterobifunctional cross-linker, GMBS (2 mM) for 1 hour at room temperature. The excess GMBS was removed by centrifugation in microcentrifuge tubes at 10,000 rpm for 10 min. The DNA was rinsed several times with PBS. Lastly, the DNA-GMBS was incubated with the antibody anti-FcγRII/RIII monoclonal antibody for 2 hours at room temperature. Figure 2 illustrates the fusion reaction using GMBS. The DNA-GMBS-antibody complex (DAPLEX) was applied to a G-10 column equilibrated in PBS. Seven fractions were collected, with fractions 2 and 3 showing increased absorbance at the wavelength of 260. Protein G was then used to absorb the DAPLEX. Fractions 2 and 3 were pooled and 0.25 μg and 0.5 μg of this material was added to 10 μl (20 μg) of protein G. After incubation at 4°C for two hours, the protein G was pelleted and the supernatant removed and set aside for analysis. The protein G pellet was resuspended in 10 μl of protein loading buffer containing SDS, and analyzed along with supernatant by agarose gel electrophoresis with ethidium bromide staining. The results revealed that the supernatant contained mostly DNA and that very little DNA was associated with the pelleted fraction (Figure 3). This suggested that the conjugation efficiency between the DNA and monoclonal antibody was extremely poor.



Figure 2

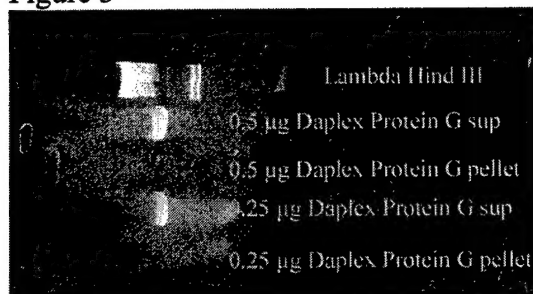
GMBS

DEN-1 DNA

QuickTime™ and a  
TIFF (Uncompressed) decompressor  
are needed to see this picture.

anti-FcγR

Figure 3



The reason for the poor efficiency is not clear. One possibility is that the reaction to add the thiol group to the DNA vaccine failed. We will use an alternative method to add the thiol group. Vector Labs' 5' EndTag Nucleic Acid Labeling System (MB-9001) will be used to thiol-label the 5' end of the linearized DNA vaccine plasmid. The thiol-labeled DNA will then be cross-linked to Maleimide-Biotin (Vector Labs, SP-1501), a compound similar to GMBS. Material from the DNA-Maleimide-Biotin fusion reaction will be subjected to agarose gel electrophoresis and transferred to a nitrocellulose membrane. The membrane will be probed using an alkaline phosphatase-streptavidin chemiluminescence kit. If a DNA-Maleimide fusion product is detected, The DNA vaccine reaction using GMBS will be repeated using the 5' EndTag Nucleic Acid Labeling System to add the thiol group. Parallel reactions will be performed substituting Maleimide (without Biotin) for GMBS. Excess linker (GMBS and/or Maleimide), unbound, DNA and unbound antibody will be removed from the reaction using Sephacryl 1000SF size exclusion columns. The resulting DAPLEX will be transferred to nitrocellulose membranes and probed for DNA. The DAPLEX will also be evaluated for binding to Raw 264.7 cells.

2. Co-immunize mice with DNA vaccine and aluminum phosphate and tetanus toxoid. Immunize mice with DNA vaccine linked with anti-FcγRI antibody.

Pilot mouse studies were conducted to evaluate the effect of aluminum phosphate (AP) on the immunogenicity of a DEN-1 DNA vaccine. Groups of six mice were immunized with 50 µg or 5 µg of DNA vaccine with or without 50 µg AP. The vaccines were administered both intramuscularly (IM) and intradermally (ID). The mice were primed and boosted on day 30.



Murine studies were initiated to evaluate the effects of tetanus toxoid (TT) on the immunogenicity of the dengue 1 DNA vaccine. Nine groups of five mice each were given various doses of DNA vaccine and TT as outlined in Table 1. Mice were primed and boosted on day 30. Serum samples were obtained at one, two and three months after administration of the last boost.

**Table 1**

A (50 $\mu$ g ID)  
B (50 $\mu$ g + 0.1 $\mu$ g TT IM)  
C (50 $\mu$ g + 1.0 $\mu$ g TT IM)  
D (50 $\mu$ g + 10 $\mu$ g TT IM)  
E (5 $\mu$ g + 0.1 $\mu$ g TT IM)  
F (5 $\mu$ g + 1.0 $\mu$ g TT IM)  
G (5 $\mu$ g + 10 $\mu$ g TT IM)  
H (5 $\mu$ g IM)  
I (50 $\mu$ g IM)

Murine studies to evaluate the immunogenicity of the DAPLEX will be initiated once efficient conjugation of the anti-Fc $\gamma$ R antibody to the DEN-1 DNA is achieved.

### 3. Analyze antibody and cellular immune responses to the adjuvanted DNA vaccines.

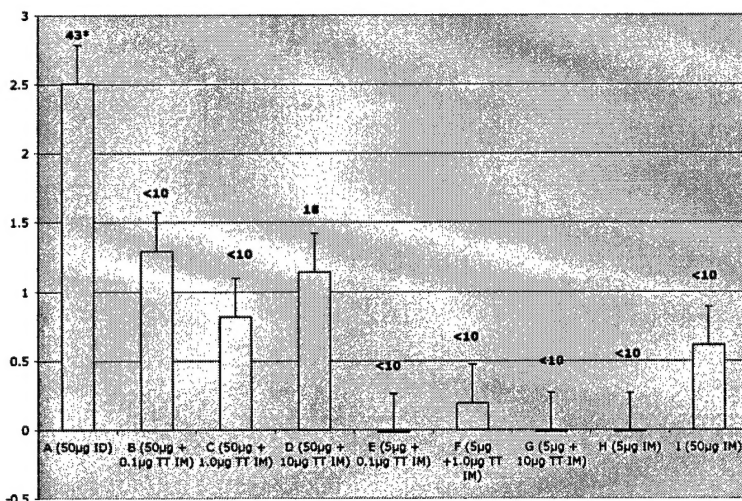
Mice immunized with the DEN-1 DNA vaccine with and without AP. To accomplish this, sera were screened for the presence of anti-dengue antibody using standard indirect ELISA as described previously [1] except that purified DEN-1 virus particles (West Pac strain) were used as antigen. Serum samples were diluted 1:100 in PBS, added to the plates and incubated at 37°C for one hour. The plates were washed and bound antibody was detected using peroxidase-labeled anti-mouse Ig antibody. At one month post-boost, there was no difference in anti-DEN neutralizing antibody levels between to AP group and the non-AP group when the vaccine was administered ID (Table 2). Aluminum phosphate did appear to slightly enhance the DNA vaccine when given IM. Earlier published studies showed that <12  $\mu$ g of DEN DNA vaccine failed to elicit an ELISA antibody response [2]. When suboptimal doses of DNA vaccine (5 $\mu$ g) were given with AP, the neutralizing antibody titers were higher when the ID route was used compared to the IM route. Overall, aluminum phosphate had a minimal affect on the immunogenicity of the DEN-1 DNA vaccine as evidenced by the lack of improvement in neutralizing antibody when the vaccine was administered by the optimal route at the highest dose.

**Table 2**

VACCINE	ROUTE	DAY 45	DAY 57
50 µg I012D1 + 50 µg AP	IM	35	40
50 µg I012D1	IM	15	<10
5 µg I012D1 + 50 µg AP	IM	<10	13
50 µg I012D1 + 50 µg AP	ID	160	290
50 µg I012D1	ID	190	270
5 µg I012D1 + 50 µg AP	ID	65	70
Vector + 50 µg AP	IM	<10	<10
Vector + 50 µg AP	ID	<10	<10

A second study was initiated to evaluate the ability of TT to enhance antibody responses to the DEN-1 DNA vaccine. Analysis of sera obtained from mice at day 90 (approximately 60 days after the last immunization) revealed all doses of TT appeared to enhance anti-dengue ELISA antibody responses, compared to DNA alone, in all groups of animals immunized with 50 µg of DEN-1 DNA vaccine (Figure 4). At the lower dose of DNA vaccine (5 µg), there appeared to be very little effect.

Plaque reduction neutralization tests (PRNT) were performed to assess neutralizing antibody responses to the DEN-1 DNA vaccine and evaluate if adding TT had any effect. Samples for this analysis were obtained at day 120 (90 days after the last immunization). Sera from the mice in each group (n=6) were pooled and tested at serial two-fold dilutions starting at 1:20 and ending at 1:160. The assay was performed as described previously [3] with the 50% endpoint being calculated using log probit analysis (PRNT50). Anti-DEN-1 neutralizing antibodies were only detected in the groups that received the DEN-1 DNA vaccine ID and the DEN-1 DNA vaccine together with 10 µg of TT IM (reciprocal PRNT50 titers of 43 and 18, respectively). This indicated that there was some enhancement of the neutralizing antibody responses by TT. The responses however were not better than DNA alone when given ID.

**Figure 4**

\* Reciprocal PRNT50 titers.

Sera from the animals were analyzed to determine if immune responses were also generated against TT. To accomplish this, 96-well plates were coated with tetanus C fragment recombinant protein [4] at 50 ng per well, and reacted with mouse sera at a dilution of 1:100. Bound antibody was detected using peroxidase-labeled anti-mouse Ig. Anti-TT immune responses were detected by ELISA in all groups receiving TT (all doses) plus DNA vaccine (data not shown). OD values ranged from 3.5 to 3.8. No quantitative comparisons can be made between the anti-TT and anti-DEN-1 immune responses since both the ELISA assays used were used only for qualitative analysis of antibody responses. Work to evaluate anti-DEN cellular immune studies by ELISPOT is ongoing.

## **Year 2**

### **Summary Statement of Work**

1. Immunize mice 10 µg DNA vaccine and 10-fold increasing concentrations of aluminum phosphate and tetanus toxoid.
2. Analyze antibody response by indirect ELISA and plaque reduction neutralization tests. Perform ELISPOT assays to detect T-cells producing IFN $\gamma$  and IL-4 to analyze cellular immune responses.
3. Measure uptake and expression of DEN DNA vaccine formulations in vivo.

### **Progress**

Earlier studies indicated that little to no adjuvant effect was seen using 50 µg of aluminum phosphate when given together with 50 µg of DNA vaccine, although some benefit was observed if the IM route of administration was used. In light of these findings, further mouse dose titration studies were not felt to be warranted at this time. Studies to examine the uptake and expression of the DNA vaccines in vivo, with and without adjuvants, will be initiated upon production of an immunogenic experimental lot of DAPLEX.

## **Year 3**

### **Statement of Work**

1. Immunize monkeys with two DEN DNA vaccine formulations that were shown to be the most potent in murine studies. If all three formulations show similar potency, then four additional monkeys will be added so that all three DNA vaccine formulations can be evaluated.
2. Analyze antibody responses to the vaccines using ELISA and plaque reduction neutralization tests. ELISPOT assays to detect T-cells producing IFN $\gamma$  (Th1 response) and IL-4 (Th2 response) will be used to analyze cellular immune responses.
3. At six months post-priming, challenge all monkeys with a low passage live DEN-2 virus to evaluate protective efficacy.

### **Progress**

Non-human primate studies will begin upon completion of the murine work.

## KEY RESEARCH ACCOMPLISHMENTS

- Anti-FcγR antibodies that recognize P388D1 and Raw 264.7 cells were identified.
- P388D1 cells failed to express prM and E proteins from the linear DNA vaccine plasmid but the Raw264.7 cells efficiently expressed the proteins. The Raw 264.7 cells will be used in the in vitro binding studies.
- Aluminum Phosphate was demonstrated to slightly enhance anti-dengue neutralizing antibody responses, only when the DNA vaccine was given ID at suboptimal doses. There was essentially no effect when the vaccine was administered IM.
- The antibody responses to the DEN 1 DNA vaccine given IM were enhanced slightly by combining it with TT. The ELISA and neutralizing antibody levels were inferior to the levels obtained with ID administration of the vaccine alone. The Animals also responded to the TT component as illustrated by the detection of anti-TT antibodies by ELISA.

## REPORTABLE OUTCOMES

Studies conducted in mice to date demonstrated that the addition of AP and TT to a DEN-1 DNA vaccine study results in slight improvement in antibody responses when administered IM.

## CONCLUSIONS

TT and AP appear to have some adjuvant effect on the neutralizing antibody responses to a DEN-1 prME DNA vaccine when given IM. These antibody responses however were not superior to the antibody responses elicited when the same DNA vaccine was given alone by the ID route.

Anti-FcγRII/III monoclonal antibodies were found that recognize Raw 264.7 mouse monocyte/macrophage cells. The efficiency of linking the monoclonal antibodies to linearized DNA vaccine was poor but may be improved using an alternative method of attaching the thiol group to the 5' end of the vaccine to produce the dengue 1 DNA vaccine-antibody complex (DAPLEX). Following demonstration of in vitro binding and expression of the DAPLEX in Raw 264.7 cells, murine studies of immunogenicity will be conducted.

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